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Involvement of Integrin $\alpha_v\beta_3$ and Cell Adhesion Molecule L1 in Transendothelial Migration of Melanoma Cells

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Tumor metastasis involves many stage-specific adhesive interactions. The expression of several cell adhesion molecules, notably the integrin $\alpha_v\beta_3$, has been associated with the metastatic potential of tumor cells. In this study, we used a novel in vitro assay to examine the role of $\alpha_v\beta_3$ in the transmigration of melanoma cells through a monolayer of human lung microvascular endothelial cells. Confocal microscopy revealed the presence of the integrin $\alpha_v\beta_3$ on melanoma membrane protrusions and pseudopods penetrating the endothelial junction. $\alpha_v\beta_3$ was also enriched in heterotypic contacts between endothelial cells and melanoma cells. Transendothelial migration of melanoma cells was inhibited by either a cyclic Arg-Gly-Asp peptide or the anti-$\alpha_v\beta_3$ monoclonal antibody LM609. Although both platelet endothelial cell adhesion molecule-1 and L1 are known to bind integrin $\alpha_v\beta_3$, only L1 serves as a potential ligand for $\alpha_v\beta_3$ during melanoma transendothelial migration. Also, polyclonal antibodies against L1 partially inhibited the transendothelial migration of melanoma cells. However, addition of both L1 and $\alpha_v\beta_3$ antibodies did not show additive effects, suggesting that they are components of the same adhesion system. Together, the data suggest that interactions between the integrin $\alpha_v\beta_3$ on melanoma cells and L1 on endothelial cells play an important role in the transendothelial migration of melanoma cells.

INTRODUCTION

The process of tumor metastasis consists of a complex cascade of adhesive interactions between tumor cells and host tissues (Nicolson, 1988; Stetler-Stevenson *et al.*, 1993; Orr *et al.*, 2000). The endothelium of blood vessels constitutes a physical barrier to cells in the circulatory system and metastatic cells must penetrate the interendothelial junctions to invade the underlying tissue. Although much is known about the adhesive interactions during the invasion of the basement membrane, relatively little is known about the mechanism by which tumor cells pass through the endothelial junction. We have developed a novel in vitro coculture assay to investigate the molecular interactions and morphological changes during cancer cell extravasation (Sandig *et al.*, 1997; Voura *et al.*, 1998a). Our previous work has shown that although platelet-endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial-cadherin are not required for melanoma transendothelial migration, heterotypic interactions with classic cadherins may play a role in this process (Sandig *et al.*, 1997). However, inclusion of anti-cadherin antibodies in the coculture assay results in only a low level of inhibition of the transmigration process, implicating the involvement of other cell adhesion molecules in this process.

In this report, we examine the role of $\alpha_v\beta_3$ during melanoma transendothelial migration in our in vitro assay system. The integrin $\alpha_v\beta_3$ was first identified as the "vitronectin receptor" but will adhere to a host of other extracellular matrix (ECM) proteins, including fibronectin, laminin, collagen, and osteopontin (Smith and Cheresh, 1990; Horton, 1997). $\alpha_v\beta_3$ is known to promote cell attachment and spreading, as well as cell locomotion (Seftor *et al.*, 1992; Danen *et al.*, 1994). Expression of $\alpha_v\beta_3$ by melanoma cells has been linked to the progression of disease (Albelda *et al.*, 1990; Felding-Habermann *et al.*, 1992; Danen *et al.*, 1994; Weterman *et al.*, 1994; Natali *et al.*, 1997; Johnson, 1999). In addition to mel-

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Abbreviations used: ECM, extracellular matrix; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; HMVEC, human microvascular endothelial cell; Ig, immunoglobulin; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PECAM-1, platelet endothelial cell adhesion molecule-1.

anoma, the α_v integrins have been implicated in invasion and metastasis of other forms of cancers (Lafrenie *et al.*, 1994; Yun *et al.*, 1996).

$\alpha_v\beta_3$ undergoes heterophilic binding with PECAM-1 and L1 (Buckley *et al.*, 1996; Montgomery *et al.*, 1996). Both PECAM-1 and L1 are members of the immunoglobulin (Ig) superfamily of cell adhesion molecules. PECAM-1 is expressed in high levels on endothelial cells and is concentrated in the endothelial junctions (Albelda *et al.*, 1991). It has been shown to play a role in the transendothelial migration of leukocytes (Muller *et al.*, 1993; Muller, 1995). L1 is expressed primarily in the nervous system. However, a nonneuronal form of L1 is found on leukocytes, epithelial cells, and various cancer cells (Kowitz *et al.*, 1992; Kujat *et al.*, 1995; Pancook *et al.*, 1997). L1 contains six Ig-like domains and five fibronectin type III-like repeats (Hortsch, 1996). In addition to homophilic binding (Miura *et al.*, 1992; Zhao and Siu, 1995, 1996), L1 interacts with $\alpha_v\beta_3$ and other integrins through an RGD sequence in its Ig-6 domain (Ruppert *et al.*, 1995; Montgomery *et al.*, 1996; Felding-Habermann *et al.*, 1997; Yip *et al.*, 1998, Yip and Siu, 2001). L1 also interacts heterophilically with laminin (Hall *et al.*, 1997) and other Ig-like molecules (Kuhn *et al.*, 1991; Horstkorte *et al.*, 1993).

L1 is shed from melanoma cells and has been suggested to provide an adhesive matrix for these cells via cell bound $\alpha_v\beta_3$ (Montgomery *et al.*, 1996). Ligation of $\alpha_v\beta_3$ with L1 also promotes haptotaxis of melanoma cells. Likewise, soluble L1 has been shown to provide an adhesive matrix for glioma cells (Izumoto *et al.*, 1996). However, the precise roles of $\alpha_v\beta_3$ and L1 during cancer metastasis are not known. We have, therefore, used our in vitro model of melanoma cell transendothelial migration to determine whether melanoma $\alpha_v\beta_3$ integrin-mediated interactions with L1 are involved in the process. Our results show that $\alpha_v\beta_3$ is enriched in the heterotypic contacts between melanoma cells and endothelial cells. Transendothelial migration of melanoma cells is inhibited by Arg-Gly-Asp peptides and antibodies against $\alpha_v\beta_3$ and L1.

MATERIALS AND METHODS

Cells and Culture Conditions

Human microvascular endothelial cells (HMVECs) were purchased from Clonetics (San Diego, CA). HMVECs were cultured in EGM-2MV medium (Clonetics), containing 100 U of penicillin and 100 μg of streptomycin (Life Technologies, Gaithersburg, MD) per milliliter of EGM medium. The human melanoma cell line WM239 was a gift from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). WM239 cells, as well as the M21 melanoma cell lines (M21, M21-L, M21-L12, and M21-L4) (Felding-Habermann *et al.*, 1992; Montgomery *et al.*, 1996), were cultured in RPMI-1640 medium prepared by the Ontario Cancer Institute Media Kitchen (Toronto, Ontario, Canada). The RPMI-1640 medium was supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) and 10% fetal bovine serum (Life Technologies). All cells were maintained in a humidified 37°C atmosphere containing 5% CO_2.

Antibodies and Peptides

The function-blocking LM609 monoclonal antibody (mAb) against $\alpha_v\beta_3$ was kindly provided by Dr. David Cheresh (Scripps Research Institute, La Jolla, CA) (Cheresh and Spiro, 1987). The rabbit antibodies recognizing L1 Ig-1–3, Ig-4–6, and fibronectin repeats were generated against recombinant L1 proteins (Zhao and Siu, 1995). The mAb P2B1 against CD31 (Ashman *et al.*, 1991) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA). This antibody was used as a nonblocking control mAb in the antibody inhibition studies.

The linear RGD peptide (PSITWRGDGRDLQEL) and the control RAD peptide (PSITWRADGRDLQEL) were synthesized based on the human L1 RGD sequence in the sixth Ig-like domain (Ig6) (Yip *et al.*, 1998). The cyclic RGD (cyclo-RGDfV) peptide and the cyclic RAD (cyclo-RADfV) peptide were purchased from Peptides International (Louisville, KY).

Transendothelial Migration Assays

The in vitro transendothelial migration assay was carried out as previously described (Sandig *et al.*, 1997; Voura *et al.*, 1998b). Round glass coverslips (12 mm in diameter and 0.1 mm in thickness) (Fisher Scientific, Fair Lawn, NJ) were coated Matrigel (Becton Dickinson, Bedford, MA). The Matrigel was diluted 1:8 in ice-cold water and applied to prechilled coverslips in 24-well plates (Flow Laboratories, McLean, VA). Matrigel (200 μl) was added to each well. Exactly 100 μl was subsequently removed and the remaining volume was air-dried overnight in a sterile flow hood at room temperature. Coverslips were rehydrated in Hanks' buffered saline solution. The Matrigel formed a thin layer of ECM to support HMVEC attachment and the formation of a monolayer of endothelial cells that mimics the endothelium. Coverslips were transferred to 35-mm-diameter dishes. Medium (~200 μl) containing 1–1.5 $\times$ 10^5 HMVECs (passage 5–9) was placed on each coated coverslip. Cells were allowed to settle for 3–4 h. Coverslips were then carefully transferred to a new 24-well plate and incubated in the EGM medium with 10 ng/m1 tumor necrosis factor-α (Life Technologies). After 12 h, melanoma cells were added to the HMVEC monolayer.

Melanoma cells were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) by incubation in 12.5 μg/ml DiI for 10 min at 37°C. The DiI-labeled cells were washed three times in Hanks' buffered saline solution and resuspended at 2.4 $\times$ 10^6/ml HMVEC medium. Then, 25 μl of labeled cells was added to the monolayer. For inhibition experiments, antibodies, and peptides were added to the HMVEC monolayers for 30 min before the addition of melanoma cells. For those experiments examining the inhibitory effects of antibodies on each cell type individually, the cells were preincubated with the inhibitor and then washed before coculture. The mAb P2B1 against PECAM-1/CD31, which does not interfere with the function of PECAM-1, was used as the control in these experiments. Preincubations involving HMVECs were carried out by adding the reagent or phosphate-buffered saline (PBS) to the monolayer after the overnight culture. The unbound material was removed by washing. The coculture was carried out for 1, 3, and 5 h at 37°C before fixation and staining of F-actin for epifluorescence microscopy. In all inhibition studies, the total number of melanoma cells associated with the HMVEC monolayer was estimated for all coverslips to ensure that any reduction in the number of transmigrated cells was not due to an impairment of cell attachment.

Immunofluorescence Staining of Cells

To label F-actin, cells were fixed with the use of 3.5% (wt/vol) paraformaldehyde at room temperature for 5 min. These cells were washed three times for 3 min each in PBS, pH 7.4, and then extracted for 5 min in cytoskeleton-stabilizing buffer, pH 6.9, containing 0.1 M 1,4-piperazine-*N,N*′,-bis(2-ethanesulfonic acid), 1 mM EGTA, 4% (wt/vol) polyethylene glycol 8000, and 0.1% Triton-X 100. The extraction procedure was followed by another series of washes and a 5-min blocking step in 1% (wt/vol) bovine serum albumin (BSA). Cells were labeled in a 1:10 dilution of dipyrromethene boron difluoride-fluorocein (BODIPY-FL) phallacidin (Molecular Probes) in blocking solution for 45 min at room temperature. Coverslips were then washed three times for 3 min each in

PBS. Strips cut from plastic coverslips were used as spacers (stacked 2 high) when the coverslips were mounted on microscope slides in a mounting medium composed of 80% glycerol and 2.5% (wt/vol) 1,4-diazabicyclo-[2,2,2]-octane as an antibleaching agent (Sigma, St. Louis, MO) in PBS. The preparations were sealed with nail enamel and then subjected to epifluorescence microscopy.

To label the integrin $\alpha_v\beta_3$, cells on coverslips were fixed with 100% methanol, which was prechilled to −20°C. After three washes, cells were incubated in the blocking solution for 5 min. The anti-$\alpha_v\beta_3$ mAb LM609 was diluted 1:100 in blocking solution and added to coverslips. After 45 min of incubation at room temperature, cells were washed three times and then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Sigma), which was diluted 1:300 in blocking solution. Incubation was carried out for 45 min at room temperature. The coverslips were washed and then mounted for confocal microscopy.

Staining of the L1 was carried out with the use of a rabbit antiserum increased against the recombinant protein that contained the five fibronectin type III-like repeats of L1 (Zhao and Siu, 1995). Cells were fixed with 3.5% paraformaldehyde. After blocking with 1% BSA, cells were incubated with the primary antiserum at a dilution of 1:100 for 45 min at room temperature. The coverslips were washed and then incubated for another 45 min with Texas Red-conjugated or fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies at a dilution of 1:300. The coverslips were washed and then mounted for confocal microscopy.

For double staining of L1 and $\alpha_v\beta_3$ integrin, coverslips of cocultures were fixed in 3.5% (wt/vol) paraformaldehyde in PBS at room temperature for 15 min. The coverslips were blocked with 1% (wt/vol) BSA for 5 min and then incubated for 1.5 h with the primary antibodies as described above. After washing, coverslips were incubated with secondary antibodies with the use of a 1:200 dilution of Alexa 488 goat anti-mouse and Alexa 598 goat anti-rabbit (Molecular Probes) in 1% BSA in PBS for 1 h. Coverslips were washed and mounted for confocal microscopy.

Laser Scanning Confocal Microscopy

Laser scanning confocal microscopy was carried out with the use of an MRC 600 confocal imaging system (Bio-Rad, Richmond, CA) on a Nikon Optiphot microscope, equipped with a 60× objective. Alternatively, a Zeiss Axiovert 135 inverted microscope equipped with a 63× Neofluor objective and an LSM 410 confocal attachment was used. Serial optical sections were routinely taken at 1-μm thickness in an apical-to-basal direction.

Quantification of Transmigration by Melanoma Cells

Quantitative analysis of the melanoma cell transmigration was carried out by epifluorescence microscopy with the use of a Wild Leitz Orthoplan universal large-field microscope equipped with a 25× objective. All experiments were done in triplicates unless indicated otherwise. Melanoma cells associated with the endothelium were separated into three stages of transmigration according to the morphological criteria of Voura *et al.* (1998a): 1) round cells attached on the endothelium, 2) cells showing clear signs of penetration into the endothelial junctions and those intercalated between endothelial cells, and 3) cells spreading underneath the endothelium and those invading the Matrigel. Melanoma cells in category 3 were taken to be transmigrated cells.

Three sets of 15 fields were scored for each coverslip to account for any preferential accumulation of melanoma cells in certain areas of the coverslip. Each set of 15 fields usually contained >100 melanoma cells. In triplicate experiments, >1000 cells were examined and scored for any one time point. All cell counts were carried out on F-actin–stained preparations with the melanoma cells preloaded with DiI for identification. Selected coverslips were also examined by laser scanning confocal microscopy to confirm the relative distribution of melanoma cells in all three categories.

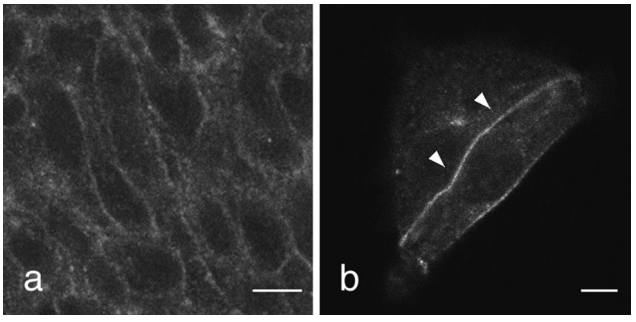

Figure 1. Confocal images showing the distribution of $\alpha_v\beta_3$ in HMVEC and WM239 melanoma cells. Cells were fixed with cold methanol and immunofluorescence staining was carried out with the use of mAb LM609 directed against $\alpha_v\beta_3$ integrin. (a) $\alpha_v\beta_3$ staining of a monolayer of HMVECs cultured on Matrigel. (b) WM239 cells showing an enrichment of $\alpha_v\beta_3$ staining at the cell-cell contact region (arrowheads). Bars, 10 μm.

RESULTS

Enrichment of $\alpha_v\beta_3$ in Heterotypic Contacts between Melanoma Cells and Endothelial Cells

As a first step to examine the role of the integrin $\alpha_v\beta_3$ in the transendothelial migration of melanoma cells, we examined the distribution of $\alpha_v\beta_3$ on both HMVEC and WM239 melanoma cells. Immunofluorescence labeling experiments were carried out with the use of the anti-$\alpha_v\beta_3$ mAb LM609 (Figure 1). The overall $\alpha_v\beta_3$ staining was relatively weak in HMVECs and was mainly associated with the plasma membrane. WM239 melanoma cells also expressed $\alpha_v\beta_3$ primarily on the cell membrane and a higher concentration of $\alpha_v\beta_3$ was present in the cell-cell contact regions.

To examine the distribution of $\alpha_v\beta_3$ during extravasation of melanoma cells, cocultures of WM239 cells and HMVECs were labeled with the anti-$\alpha_v\beta_3$ mAb LM609 and series of optical images in the X/Y plane were taken for further analysis (Figure 2). To distinguish melanoma cells from endothelial cells, WM239 cells were preloaded with DiI before seeding on the HMVEC monolayer. Before extravasation, diffuse $\alpha_v\beta_3$ staining was observed on the entire melanoma cell membrane. The first sign of invasion through the endothelial junction was the formation of membrane blebs from the basolateral regions of the attached melanoma cells. These membrane protrusions eventually formed a pseudopod, which penetrated into the endothelial junction. Both blebs and pseudopods generally showed stronger $\alpha_v\beta_3$ staining, suggesting the presence of a higher concentration of $\alpha_v\beta_3$ on these membrane protrusions (Figure 2A). On the retraction of neighboring HMVECs, the transmigrating WM239 cell became intercalated between endothelial cells. $\alpha_v\beta_3$ staining was clearly associated with the heterotypic contacts between melanoma cells and the surrounding endothelial cells, whereas staining of the homotypic contact regions between endothelial cells was much weaker (Figure 2B). These images thus indicate an enrichment of $\alpha_v\beta_3$ in the contact regions between melanoma cells and endothelial

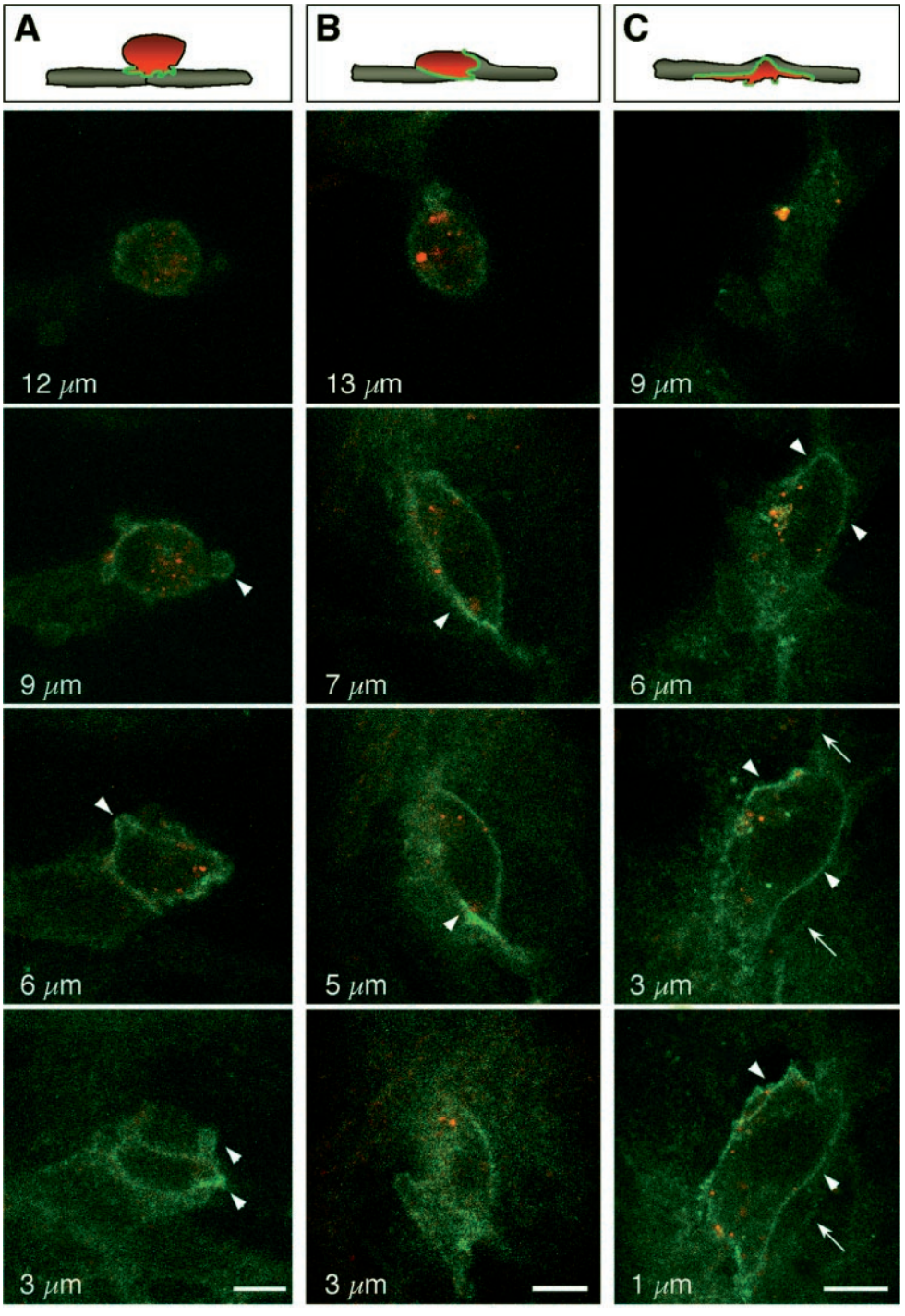

Figure 2. Confocal series showing an enrichment of $\alpha_v\beta_3$ on membrane protrusions of melanoma cells and in heterotypic contacts during transendothelial migration. DiI-labeled melanoma cells were seeded on top of an HMVEC monolayer and fixed at different times of coculture. Coverslips were stained with the use of mAb LM609 and serial optical images were taken at 1-μm thickness. A schematic drawing is shown at the top of each series. Individual images are shown in an apical-to-basal direction and labeled with its distance from the bottom of the endothelium. (A) An optical series showing a melanoma cell at the initial stage of invasion through the endothelium. Membrane protrusions sent from the basolateral surfaces of the melanoma cells were labeled with $\alpha_v\beta_3$ (arrowheads). (B) An optical series showing a spindle-shaped melanoma cell intercalated between two endothelial cells. The heterotypic contacts were enriched in $\alpha_v\beta_3$, especially at the leading edge of an endothelial cell spreading on top of the melanoma cell (arrowheads). (C) An optical series showing a transmigrated melanoma cell spreading on the Matrigel under the endothelium. An enrichment of $\alpha_v\beta_3$ persisted in the heterophilic contact regions in all the X/Y sections (arrowheads). In comparison, the endothelial junctions were only weakly stained (arrows). Bars, 10 μm.

cells. Also, endothelial cells spreading on top of a transmigrating melanoma cell often displayed strong $\alpha_v\beta_3$ staining in the leading edges. A higher concentration of $\alpha_v\beta_3$ persisted in the heterotypic contacts of melanoma cells spreading on the Matrigel (Figure 2C). These results suggest that the integrin $\alpha_v\beta_3$ plays an important role throughout the transmigration process of melanoma cells.

Inhibition of Melanoma Cell Transendothelial Migration by an Anti-$\alpha_v\beta_3$ Antibody

Given that $\alpha_v\beta_3$ was found in the heterotypic contacts during melanoma cell transmigration, we next examined the effects of the function-blocking mAb LM609 on melanoma cell transendothelial migration. When the antibody was added to the cocultures, melanoma transendothelial migration was reduced by 40–50% at 5 h (Figure 3A). The inclusion of a nonblocking control mAb in the assay did not result in any inhibition. The antibody did not affect the attachment of WM239 cells, because comparable numbers of melanoma cells were found associated with the HMVEC monolayer in cocultures incubated either in the presence or absence of LM609.

Because both melanoma and endothelial cells express $\alpha_v\beta_3$, we next determined whether $\alpha_v\beta_3$ molecules expressed on both cell types were involved equally in the transmigration process of melanoma cells. To address this issue, either melanoma cells or endothelial cells were preincubated with mAb LM609 and then washed to remove unbound antibody before coculture. Preincubation of the WM239 cells resulted in a 40% reduction in the number of transmigrated cells at 5 h (Figure 3B). In contrast, no significant inhibition of WM239 cell transendothelial migration was observed when HMVECs were preincubated with the antibody. The results thus indicate that the integrin $\alpha_v\beta_3$ on melanoma cells, and not endothelial cells, is involved in the transmigration process.

$\alpha_v\beta_3$-Negative Melanoma Cells Are Impaired in Transendothelial Migration

The key role of $\alpha_v\beta_3$ expressed by melanoma cells in the transendothelial migration process was further evaluated with the use of M21 melanoma cell variants either expressing or lacking the α_v subunit (Cheresh and Spiro, 1987; Felding-Habermann *et al.*, 1992). The cell line M21-L does not synthesize α_v and lacks $\alpha_v\beta_3$. The M21-L4 cell line (derived from M21-L cells transfected with the α_v cDNA) expresses $\alpha_v\beta_3$, whereas the M21-L12 line (derived from mock transfectants) does not. The ability of these cell lines to undergo transendothelial migration was examined (Figure 4). Whereas the α_v-positive cell lines M21 and M21-L4 showed the normal kinetics of transmigration with levels comparable with that of WM239 cells, the α_v-negative variants M21-L and M21-L12 were compromised in their ability to undergo transendothelial migration with <15% of cells transmigrated by 5 h. These results demonstrate that the transendothelial migration of melanoma cells is dependent on the its expression of $\alpha_v\beta_3$.

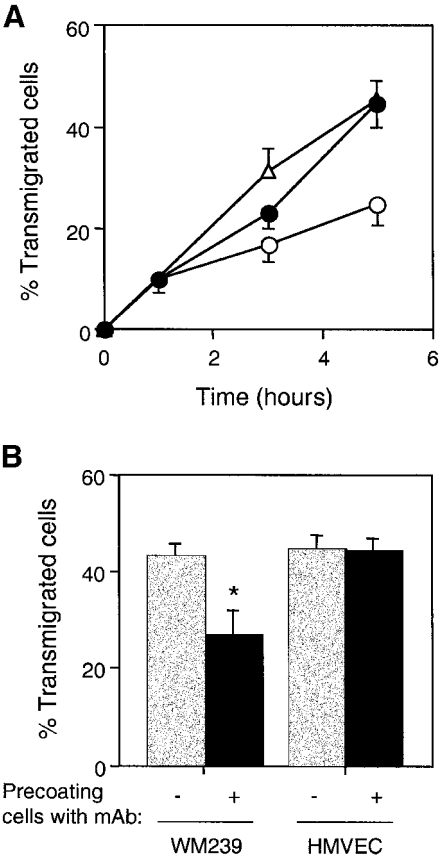

Figure 3. Inhibition of melanoma transmigration through HMVECs by mAb LM609 directed against $\alpha_v\beta_3$. (A) DiI-labeled WM239 melanoma cells were seeded on HMVEC monolayers and transmigration was allowed to occur in the presence of LM609 IgG (40 μg/ml). Coverslips were fixed at 1, 3, and 5 h of coculture. The number of transmigrated cells was scored as described in MATERIALS AND METHODS. Cocultures were incubated in the absence of antibody (△), or in the presence of mAb LM609 against $\alpha_v\beta_3$ (○) or mAb P2B1 against PECAM-1 (●). (B) Effects of preincubating cells with mAb LM609 on the transendothelial migration of melanoma cells. Either WM239 cells or HMVECs were preincubated with LM609 (40 μg/ml) for 30 min and the unbound antibody was removed. Melanoma cells were then added to the HMVEC monolayer and cocultured for 5 h. Assays were carried out with cells either precoated with mAb (solid bars) or without prior precoating (shaded bars). Data represent the mean ± SD (n = 9). The asterisk indicates a statistically significant reduction in the percentage of transmigrated cells compared with the control (Student's *t* test, $p < 0.01$).

L1 Expression in Melanoma Cells and Endothelial Cells

To which adhesion receptor on endothelial cells does the melanoma $\alpha_v\beta_3$ bind? In addition to vitronectin and other ECM components, $\alpha_v\beta_3$ is known to undergo heterophilic binding with the cell adhesion molecules PECAM-1 and L1 (Buckley *et al.*, 1996; Montgomery *et al.*, 1996). Although both PECAM-1 and L1 are expressed by HMVECs, we have found that PECAM-1 redistributes away from the endothelial junction and is not required for melanoma cell transmi-

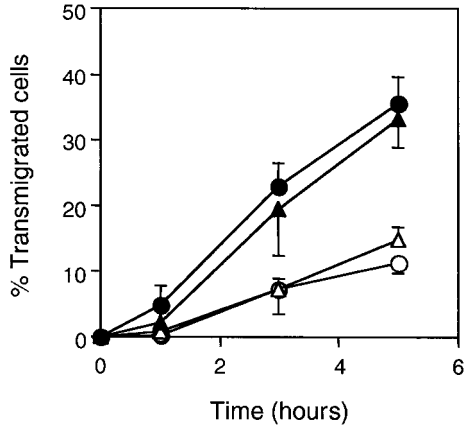

Figure 4. Transendothelial migration of the M21 melanoma cells through HMVEC monolayers. The percentages of transmigrated cells were scored at different times of coculture for M21 cells (α_v+) (●), M21-L cells (α_v−) (○), M21-L4 cells (α_v+, M21-L cells transfected with α_v cDNA (▲) and M21-L12 cells (α_v−, M21-L mock transfectant) (△). Data represent the mean ± SD (n = 9).

gration (Voura *et al.*, 2001). Therefore, our studies were focused on the potential involvement of L1. We first examined the expression of L1 in HMVECs and WM239 cells. Protein blot analysis showed that L1 was synthesized in both WM239 cells and HMVECs (Figure 5a). Whereas L1 was secreted by WM239 cells, L1 was not detected in the HMVEC-conditioned medium. Immunolocalization studies revealed several interesting features of L1 in WM239 cells (Figure 5, b and c). Whereas the plasma membrane of HMVECs showed clear staining of L1, L1 staining was barely detectable on the membrane of WM239 cells. However, perinuclear staining of L1 was evident in both types of cells, indicating that they were actively synthesizing L1. Membrane protrusions loaded with L1 were occasionally observed at the cell periphery, suggesting that L1 might be released into the medium by evagination of the plasma membrane.

Interactions between L1 and $\alpha_v\beta_3$ during transendothelial migration of melanoma cells would predict colocalization of these molecules during the transmigration process. Double immunolocalization experiments were carried out and cells at different stages of transmigration were examined. Both L1 and $\alpha_v\beta_3$ showed punctate staining along the periphery of cells. In the initial stages of cell-cell interaction, high concentrations of L1 and $\alpha_v\beta_3$ were observed in regions where lamellipodia and pseudopodia of melanoma cells were in contact with the endothelium (Figure 5, d and e), suggesting the involvement of both cell adhesion molecules in the early stage of penetration. When melanoma cells became intercalated among endothelial cells, both L1 and $\alpha_v\beta_3$ were enriched along the heterotypic contacts although their staining patterns did not show complete overlap (Figure 5, f and g).

Inhibition of Transendothelial Migration of Melanoma Cells by RGD Peptides

Peptide inhibition studies were undertaken to evaluate the role of L1-$\alpha_v\beta_3$ interaction in melanoma cell transendothelial

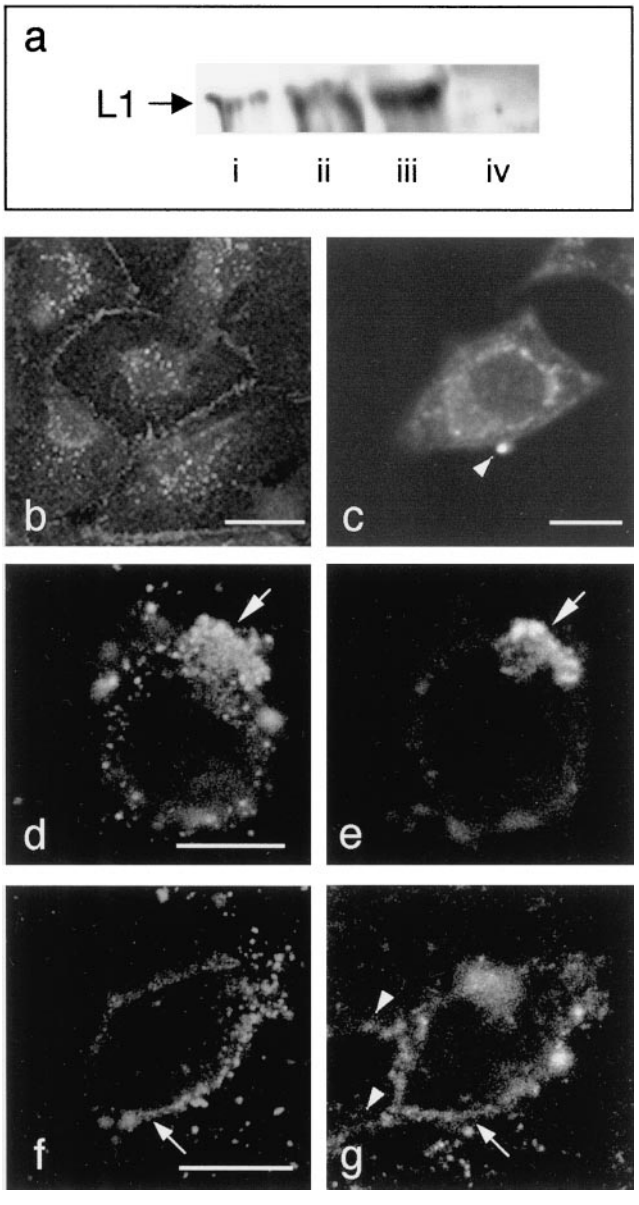

Figure 5. Expression of L1 in HMVEC and WM239 cells. (a) Protein blots stained with anti-L1 antibody (1:500 dilution): i) total cell protein of WM239 cells, ii) total cell protein of HMVECs, iii) WM239 conditioned medium, and iv) HMVEC conditioned medium. Cell were also fixed with paraformaldehyde and stained with anti-L1 antibody: HMVEC (b), WM239 cells (c). Although endothelial junctions were clearly stained with L1, the plasma membrane of melanoma cells was only weakly stained. Granules filled with L1 were occasionally observed at the cell membrane (arrowhead). Double immunostaining was carried out to examine the localization of L1 (d and f) and $\alpha_v\beta_3$ (e and g) during transendothelial migration of melanoma cells. Melanoma cells were preloaded with Hoechst dye for identification. A round WM239 cell attached on the endothelium is shown in d and e. Arrows indicate a higher concentration of L1 and $\alpha_v\beta_3$ associated with the lamellipodial structure. The staining of L1 and $\alpha_v\beta_3$ was more intense at the heterotypic contacts (arrow) than the homotypic endothelial contacts (arrowheads). A melanoma cell intercalated among endothelium cells is shown in f and g. Bars, 10 μm.

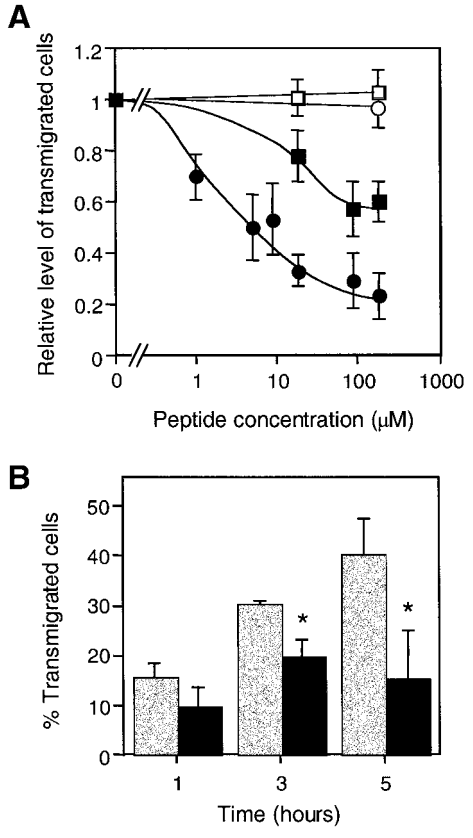

Figure 6. Inhibition of melanoma transmigration through HMVECs by RGD peptides. (A) Cocultures of WM239 cells and HMVECs were carried out in the presence or absence of different concentrations of synthetic peptides: linear RGD (■), linear RAD (□), cyclic RGD (●), and cyclic RAD (○). The percentage of transmigrated cells was determined at 5 h and then normalized to the minus-peptide control. (B) Time course experiment for the transmigration of WM239 melanoma cells in the presence of 90 μM cyclic RGD peptide (solid bars). Control assays (stippled bars) were carried out in the absence of the cyclic peptide. Data represent the mean ± SD (n = 9). The asterisks indicate a statistically significant reduction in the percentage of transmigrated cells (Student's *t* test, $p < 0.01$).

migration. The integrin $\alpha_v\beta_3$ mediates adhesion via an RGD sequence (Rouslahti and Obrink, 1996), and human L1 contains an RGD motif in 6th Ig-like domain (Ig6) (Hlavin and Lemmon, 1991). Therefore, a synthetic peptide containing the RGD motif and its flanking sequences in L1 (PSITWRGDGRDLQEL) and its control RAD peptide (PSITWR*A*DGRDLQEL) were tested for their effects on melanoma transendothelial migration. The linear RGD peptide inhibited melanoma cell transmigration by 40%, whereas the inactive RAD peptide had no effect (Figure 6A). The cyclic RGD peptide (cyclo-RGDfV) has been reported to bind $\alpha_v\beta_3$ at high affinity and block its function effectively at low concentrations (Pfaff *et al.*, 1994; Brooks *et al.*, 1996; Kerr *et al.*, 1999). Dose experiments showed that the cyclic RGD peptide was able to inhibit transendothelial migration of melanoma cells by 70%. Fifty percent inhibition was achieved at ~5 μM peptide. In contrast, the cyclic RAD peptide did not have significant effects on the transmigration of melanoma cells. Time course studies with the use of the cyclic RGD peptide (Figure 6B) indicated that, although inhibitory at all time points, the peptide had its greatest effect at 5 h of coculture. To rule out negative effects of the cyclic peptide on cell attachment, the number of melanoma cells associated with the HMVEC monolayer was estimated for all time points. Comparable numbers of cells were obtained in all cases, indicating that the RGD peptide did not affect cell attachment.

Transendothelial Migration of Melanoma Cells Involves L1 on Endothelial Cells

To determine whether L1 was directly involved in melanoma cell transmigration, we made use of a rabbit antibody raised against L1 Ig4–6, which was previously found to inhibit L1-$\alpha_v\beta_3$ interactions (Yip *et al.*, 1998). Coculture assays were carried out in the presence of these antibodies and the percentage of transmigrated cells was quantified at various time points (Figure 7A). The number of transmigrated melanoma cells was reduced by ~50% at 5 h.

Because L1 is expressed in both melanoma and endothelial cells, it is possible that L1-L1 homophilic interactions at the heterotypic contacts might play a role in the transmigration of melanoma cells. Therefore, we tested the effects of a rabbit antibody raised against the first three Ig-like domains of L1. Although this antibody is known to block L1 homophilic binding centered at the Ig2 domain (Zhao and Siu, 1995, 1996), it did not exert significant effects on the transendothelial migration of melanoma cells (Figure 7A). The data thus indicate that transendothelial migration does not involve L1-L1 homophilic binding.

These results led us to speculate that L1 on endothelial cells, and not melanoma cells, was involved in tumor cell extravasation. To address this issue, either endothelial cells or melanoma cells were preincubated with the anti-L1-Ig4–6 antibody. Cells were washed to remove unbound antibody before seeding the melanoma cells on the HMVEC monolayer. Preincubation of the WM239 melanoma cells with the antibody did not inhibit transendothelial migration (Figure 7B). However, preincubating HMVECs with the antibody did produce a small, but reproducible, level of inhibition. The number of transmigrated cells was reduced by ~20%. The data are consistent with the idea that L1 on the endothelial cells, and not the melanoma cells, has a role during transendothelial migration of melanoma cells.

The above-mentioned results suggested that L1 and $\alpha_v\beta_3$ might function as an adhesive pair during melanoma extravasation. To address this issue, we tested whether the addition of both antibodies against L1 and $\alpha_v\beta_3$ would elicit additive inhibitory effects on the transmigration process. The results showed that the inhibitory effects of these two antibodies were not additive (Figure 8). An ~40% inhibition was achieved whether the antibodies were added singly or together to the coculture. These results thus support the notion that L1 and $\alpha_v\beta_3$ are components of the same adhesive system.

DISCUSSION

In this article, we have demonstrated the involvement of integrin $\alpha_v\beta_3$ in melanoma transendothelial migration.

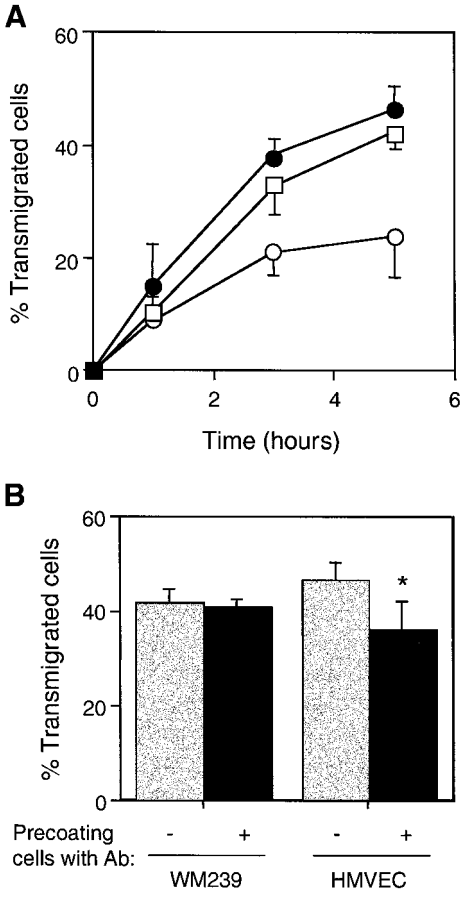

Figure 7. Effects of anti-L1 antibodies on the transendothelial migration of melanoma cells. (A) WM239 melanoma cells were added to HMVEC monolayers for transmigration in the absence of antibody (●) or in the presence of anti-L1-Ig1–3 antibody (□) or anti-L1-Ig4–6 antibody (○). (B) Effects of preincubation of cells with antibodies on WM239 cell transmigration. WM239 melanoma cells or HMVECs were preincubated with the anti-L1-Ig4–6 antibody (1:10 dilution) for 30 min at 37°C. Unbound antibodies were removed by washing before the coculture assay. The percentages of transmigrated cells were scored at 5 h. Data represent the mean ± SD (n = 9; *$p < 0.05$).

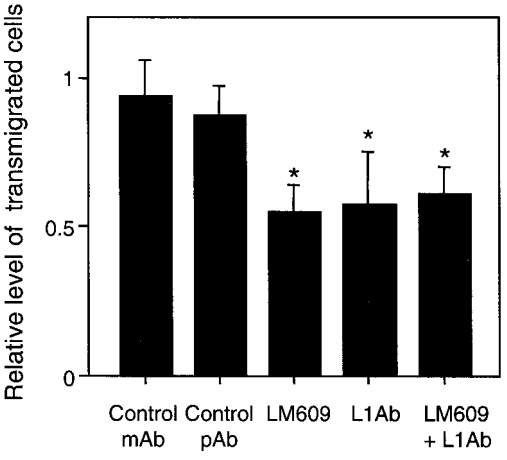

Figure 8. Inhibition of transmigration by a combination of antibodies against $\alpha_v\beta_3$ and L1. The transmigration assay was carried out in the presence of mAb LM609 (40 μg/ml) and anti-L1-Ig4–6 antibody (1:10 dilution), either singly or combined. Controls were carried out in either mAb P2B1 or rabbit preimmune serum. All values were normalized to that of the minus-antibody control. Data represent the mean ± SD (n = 9; *$p < 0.01$).

Whereas interactions between integrins and ECM are known to play an important role in the malignant behavior of melanoma cells, this is the first report to our knowledge that demonstrates a role for $\alpha_v\beta_3$ during tumor cell transendothelial migration. The expression of the integrin $\alpha_v\beta_3$ has been shown to associate with the invasiveness of a subset of tumors that eventually leave the primary tumor and cause secondary growth. Our studies have focused on the transendothelial migration process and we have found that $\alpha_v\beta_3$ on melanoma cells interacts with L1 on endothelial cells and that their interactions play a crucial role in the transmigration process.

Immunofluorescence labeling of cells in the coculture assay has revealed that $\alpha_v\beta_3$ becomes enriched in the heterotypic contacts from the initial stages of melanoma cell invasion, when membrane protrusions extending from the basolateral surfaces begin to contact the surface of the endothelium. In addition to adhesive interactions, the membrane protrusions may facilitate transmigration by releasing signaling molecules and/or proteases into the microenvironment (Basbaum and Werb, 1996; Ginestra *et al.*, 1997). A high concentration of $\alpha_v\beta_3$ persists on the contact surfaces between melanoma cells and endothelial cells throughout the transmigration process, even when the transmigrated cells are spreading on the Matrigel. $\alpha_v\beta_3$ has been found to cluster in focal contacts and to promote both cell adhesion and cell motility (Leavesley *et al.*, 1992; Seftor *et al.*, 1992; Danen *et al.*, 1994). Therefore, the ligation of $\alpha_v\beta_3$ with adhesion receptors may facilitate the passage of melanoma cells through the endothelial junction and subsequent migration on the Matrigel.

Antibody inhibition studies have demonstrated that the participation of $\alpha_v\beta_3$ is vital to the transmigration of melanoma cells. Although retardation of transmigration was observed 1 h after coculture, the inhibitory effects were most prominent at 5 h. Most melanoma cells were arrested at the stage when they became intercalated between endothelial cells. Similar data were obtained with the use of the cyclic RGD peptide to inhibit the function of $\alpha_v\beta_3$, suggesting that $\alpha_v\beta_3$ may play a more important role during the later stages of transmigration. $\alpha_v\beta_3$ is known to bind the RGD motif of a number of ECM components (Cheresh, 1987; Pfaff *et al.* 1994; Horton, 1997). Thus, antibody blocking of $\alpha_v\beta_3$ can impair the cell spreading process and delay the transmigration process.

Studies on the M21 cell line and its variants further support the role of $\alpha_v\beta_3$ during transendothelial migration of melanoma cells. Whereas the transmigration efficiency of M21 cells is comparable with that of WM239 cells, the α_v-deficient variants transmigrated poorly, correlating very well with their decreased tumorigenicity in nude mice (Felding-Habermann *et al.*, 1992). Both transmigration efficiency

and tumorigenicity are rescued when α_v expression is restored by cDNA transfection. These findings suggest that the reduction in transmigration efficiency may contribute to the decreased tumorigenicity of the α_v-deficient cells. Our previous findings (Voura *et al.*, 1998a) on the poorly metastatic WM35 melanoma cell line (Bani *et al.*, 1996) are reminiscent of the M21 variants. The migratory ability of WM35 cells, like the α_v-negative M21 cells, was depressed compared with their more aggressive counterparts. Tumor necrosis factor-α can stimulate the ability of the WM35 cells to transmigrate. However, this effect is inhibited by the anti-$\alpha_v\beta_3$ mAb LM609 (Voura and Siu, unpublished data).

Although both melanoma cells and endothelial cells express $\alpha_v\beta_3$, blocking the $\alpha_v\beta_3$ molecules on melanoma cells, and not endothelial cells, with mAb LM609 inhibits transmigration. Thus, transmigration of melanoma cells likely requires the participation of only those $\alpha_v\beta_3$ molecules expressed on melanoma cells. Although the expression of $\alpha_v\beta_3$ on endothelial cells has been implicated in endothelial cell motility and angiogenesis (Brooks *et al.*, 1994, Kim *et al.* 2000; Kumar *et al.*, 2000), endothelial $\alpha_v\beta_3$ may not be directly involved in the transmigration process of melanoma cells.

We have identified L1 as the major adhesion receptor on endothelial cells that binds $\alpha_v\beta_3$, although both L1 and PECAM-1 on endothelial cells can serve as ligands for $\alpha_v\beta_3$ (Piali *et al.*, 1995; Buckley *et al.*, 1996; Montgomery *et al.*, 1996; Felding-Habermann *et al.*, 1997). Whereas PECAM-1 has been found to play an important role in leukocyte migration (Muller, 1995), we have found that PECAM-1 is not required for the transendothelial migration of melanoma cells. In fact, PECAM-1 is redistributed away from endothelial junctions associated with transmigrating melanoma cells and is absent in the heterotypic contacts (Voura *et al.*, 2001). This leaves L1 as the major target for ligation with $\alpha_v\beta_3$ on melanoma cells. Consistent with other reports that tumor cell $\alpha_v\beta_3$ can adhere to L1-coated substrates via the RGD sequence in the L1 Ig6 domain (Ebeling *et al.*, 1996; Montgomery *et al.*, 1996; Duczmal *et al.*, 1997), RGD peptides inhibit the transmigration of melanoma cells. Our studies with the use of cells precoated with antibodies further demonstrate that it is the L1 present on endothelial cells, and not melanoma cells, that is involved in transendothelial migration. These results might explain why we did not observe complete colocalization between L1 and $\alpha_v\beta_3$ in heterotypic contacts during the transmigration process.

The expression of L1 has been found in a metastatic variant of the melanoma cell line K1735, whereas nonmetastasizing cells are L1-negative, suggesting a role for L1 in tumor progression (Linnemann *et al.*, 1989). In addition to melanoma, L1 has been found in malignant cells of diverse origin, including neuroblastoma, osteogenic sarcoma, squamous lung carcinoma, and skin carcinoma cell lines (Mujoo *et al.*, 1986; Linnemann *et al.*, 1989; Reid and Hemperly, 1992). Our morphological studies show that only a low level of L1 is associated with the plasma membrane of melanoma cells. The homophilic binding site of L1 has been mapped to a sequence in the Ig2 domain (Zhao *et al.*, 1998). Antibody blocking experiments with the use of anti-L1-Ig1–3 antibody indicate that L1-L1 pairing between melanoma cells and endothelial cells does not contribute significantly to the adhesion and transmigration of melanoma cells. Because a substantial amount of L1 is released into the medium by melanoma cells, we speculate that L1 molecules released by melanoma cells may adhere to the surface of endothelial cells and augment the binding interactions between melanoma $\alpha_v\beta_3$ and L1 on endothelial cells.

Metalloproteinases, as well as other ECM-degrading enzymes, have been shown to be important for cancer progression, and the localization of these enzymes to the surface of invasive cells is important for their function (de Vries *et al.*, 1996; Chapman, 1997; Werb, 1997; Brunner *et al.*, 1998; Deryugina *et al.*, 1998). In addition to cell adhesion and migration, $\alpha_v\beta_3$ has been found to localize matrix metalloproteinase-2 (MMP-2) to the surface of invasive cells and this interaction can be inhibited by the $\alpha_v\beta_3$ function-blocking antibody LM609 (Brooks *et al.*, 1996). The interaction of MMP-2 with $\alpha_v\beta_3$ also plays a role in tumor angiogenesis (Brooks *et al.*, 1998; Silletti *et al.*, 2001). Membrane type-1 MMP can also localize MMP-2 on the cell surface of invasive cells via tissue inhibitor of metalloproteinase-2. Therefore, inhibiting MMP-2 interaction with the $\alpha_v\beta_3$ integrin may not preclude the membrane association of the enzyme during melanoma cell extravasation (Strongin *et al.*, 1995; Werb, 1997). However, recent evidence suggests that the cooperation of both $\alpha_v\beta_3$ and membrane-type-1 MMP is required for the localization of active MMP-2 to the cell surface (Deryugina *et al.*, 2000, 2001; Hofmann *et al.*, 2000). Therefore, it is possible that a further component of our observed $\alpha_v\beta_3$ inhibition studies is provided by a reduced localized activation of MMP-2 on the surface of the melanoma cells during transmigration. Such blocking would most likely result in reduced melanoma cell spreading on the Matrigel matrix.

Our studies have established a role for $\alpha_v\beta_3$-L1 interactions during transendothelial migration of melanoma cells. However, although antibodies directed against L1 and $\alpha_v\beta_3$, as well as cyclic RGD peptides, are potent inhibitors of this process, we are unable to achieve complete inhibition with the use of these reagents. It is, therefore, likely that other adhesion receptors are involved. Our previous work has suggested the involvement of classic cadherins (Sandig *et al.*, 1997). Other junctional structures, such as gap junctions, may also be involved (El-Sabban and Pauli, 1991). Future studies will focus on the identification and characterization of additional adhesion receptors involved in this important step of cancer metastasis.

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